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Table of Contents

INTRODUCTION: Our hypothesis is that unique genes on human chromosomes are involved in the progression of breast cancer from an insulin-dependent to insulin-independent state. During breast cancer development, tumors progress from an insulin-dependent to an insulin-independent state. In order to simulate the progression of breast cancer from an insulin-dependent to insulin-independent state, our lab developed the following three cell line model system. The parental cell line that was used was CAL51, an ER-, wild type p53 carrying, metastatic breast cancer cell line that has the unique feature of being diploid in chromosome number. Additionally, CAL51 displays the tumorigenic phenotype including rapid population doubling time in the absence of insulin, anchorage-independent growth in soft agar culture, and rapid tumor formation in athymic nude mice. Through microcell-mediated chromosome transfer, chromosome 17 was introduced to create the suppressed cell line CAL 17. As stated, this cell line exhibits a suppressed phenotype contrasting CAL51 in that has an increased population doubling time, loss of anchorage-independent growth and nontumorigenicity. Our laboratory has applied the technique of retroviral insertional mutagenesis to induce insulin-independent revertant cell lines from chromosome 17-mediated suppressed breast cancer. After culturing for two months in the absence of insulin, we isolated and established 48 revertant cell sublines termed RICs (Retrovirus-induced Insulin-independent Cell) that displayed insulin-independent growth. It should be noted that while the suppressed cell line CAL17 is insulin-independent, the tumorigenic cell line, CAL51 and revertant cell lines, RICs, are insulin-independent. All three cell lines were characterized for their tumorigenicity by looking at their cell generation time, insulin-independent growth, agar clonogenicity, and tumor formation in nude athymic mice. Additionally, the involvement of insulin growth factor family members was investigated through northern and western analysis and finally, microarray analysis. These cell lines provide a useful biological resource to study breast cancer transition from insulin-dependence to insulin-independence.

BODY: Characterization of Insulin-independent Cell Sublines Derived from the Insulin-dependent Breast Cancer Cell Line CAL/17

A. Technical Objective A: To compare the *in vitro* and *in vivo* growth features among the parental insulin-independent cell line, the chromosome 17-mediated suppressed insulin-dependent cell line, and the revertant insulin-independent cell line.

Phenotypic characterization of these cell lines was undergone in order to reconfirm and establish their tumorigenicity. One such assay that was performed was the generation of growth curves for each cell line in plastic culture. Each cell line was grown under two different conditions, one set was grown only in Full medium while the other set was grown in Full medium supplemented with insulin (at 2.5ng/ml). When grown in the absence of insulin, the parental CAL51 cell line displays the fastest population-doubling curve whereas the suppressed cell line CAL17 has the slowest. The *in vitro* proliferation rates of the revertant cell sublines fell in between these two cell lines. In the presence of insulin, the population doubling time of the suppressed cell line CAL17 increased significantly over its doubling time in media without insulin. The parental and revertant cell line continued to grow in their similar rates in the presence of insulin. When the cell lines were plated in soft agar and observed for colony growth, only the parental cell line CAL51 displayed anchorage-independence, one of the hallmarks of tumorigenicity. Finally, the cell lines were injected into athymic nude mice and observed for three months for tumor growth. As previously recorded, the parental cell line CAL51 displayed tumor growth that peaked at one month. The suppressed cell line, CAL17, however, took an additional two months for substantial tumor growth to appear, thereby showing significant tumor suppressive activity. Similar tumor formation was seen in the revertant cell lines after three months, such that overall no significant tumorigenic activity was observed.

These experiments confirmed the tumorigenicity of the parental CAL51 cell line and the suppression of tumorigenicity by the chromosome 17-derived cell line CAL17 in that the obtained results are reflected in the rapid population doubling time, soft agar clonogenicity and tumor formation in nude athymic mice of the parental CAL51 cell line and the opposing results of the CAL 17 cell line. Even though their population doubling times fell between the CAL51 and CAL17 cell lines, overall, the revertants did not display major tumorigenic activity since no clones were formed in soft agar and tumors

developed at the same rate as the suppressed cell line CAL17. It is possible that these revertant cell lines are too early in the carcinogenic process. Alternatively, the genes that are affected by the retroviral insertion do not contribute directly to the tumorigenic process, but if combined with another genetic or chemical insult to the cell, could help promote carcinogenesis.

B. Technical Objective B: To identify and sequence novel genes involved in tumor suppression and transition to insulin-independence.

Using the retroviral hisD gene as a probe, Southern analysis of the three cell lines identified four independent cell sublines that contain unique retroviral insertion sites. Approximately 20 μg of genomic DNA from all three cell lines were digested with the restriction enzymes EcoRI and HindIII that cut in and outside the retroviral DNA. As expected, both the parental cell line CAL51 and suppressed cell line CAL17 mirror the negative placental DNA control in that neither contain retroviral fragments. All the revertant RIC cell sublines contain a single retroviral insertion. Through comparison of the two southern blots, one can see four unique insertion sites, specifically in lanes labeled ii-2, ii-7, ii-33 and ii-60. PCR amplification with retroviral-vector specific primers flanking the retroviral-genomic DNA insertion site and subsequent sequencing revealed that in actuality, there were three unique RIC clones. The retrovirus either inserted into a nonspecific portion of the genome or an Alu sequence. However, one RIC, ii-7, revealed an integrated retrovirus containing considerable homology to a BAC clone (clone AC005067.2) that spans the locus of chromosome 7q11-21 was found. Curiously, this band region does not encode a known gene related to the IGF family. While it is not uncommon for retroviruses to insert into random genomic DNA, the ability to discern the exact location of the retrovirus is beyond the scope of this laboratory, as it would entail looking at the overall chromatin structure. There is also the possibility that the insulin-independent clones that were generated could have arose from spontaneous mutations that are not at all attributable to the insertion of the retrovirus.

C. Technical Objective C: To compare expression profiles among the parental insulin-independent cell line, the chromosome 17-mediated suppressed insulin-dependent cell line, and the revertant insulin-independent cell line.

Involvement of the IGF family in terms of the revertant and parental cell line's insulin-independent growth activity was investigated. Twenty-three cDNA clones related to IGF family members were screened by Northern analysis. Most of the IGF members had equal, low or non-detectable expression for all the cell lines. However, two IGF family members, IGFIIR and IGFBP3, showed differential expression among the cell lines. In the case of IGFIIR, all the cell lines showed equal expression except revertant ii-60, which had a lower RNA expression. Similarly, all the cell lines displayed relatively equal expression of IGFBP3 except for a significantly lower expression in revertant ii-33. To verify the protein levels for these two genes in all the cell lines, western blots were performed. Overall, the western blots reconfirmed what was seen in the Northern blots, with some minor exceptions. For instance, in the IGFIIR western blot, there is a lowered protein level in revertant cell lines ii-33 and ii-60 as well as the parental cell line CAL51. Furthermore, in the IGFBP3 western blot, lowered protein expression can be seen in the parental cell line, and revertant cell lines ii-33, ii-60 and ii-83.

During the course of this study, all the cell lines were found to be mycoplasm-positive, thus calling into questions the results that were obtained. Several months were lost to this finding as a heroic attempt to clean them up was made using a Mycoplamsma removal kit (ICN Biomedicals, Inc., CA). Once these cells were successfully cleared of mycoplamsa contamination, PCR analysis was performed on these cell lines using primers designed from a G418 plasmid to detect the presence of chromosome 17. As expected, both the suppressed cell lines and the revertant cell lines contained a positive band whereas the parental cell line did not, thus allowing us to proceed recharacterizing these cells. Unfortunately, in vitro proliferation assays with these cells revealed that all the cells changed their growth characteristics. Preliminary microarray experiments comparing the cells before and after treatment further confirmed differences in gene expression as the pre and post-mycoplasma treatment cells separated into different clusters. The gene profiles from these cell lines are now encorporated into a gene expression database of breast cancer cell lines where they may eventually be of further use in future microarray data analysis studies (ie. used as training group for neural networks or for comparison among cell lines that share similar characteristics.)

KEY RESEARCH ACCOMPLISHMENTS:

1. Phenotypic analysis demonstrated the establishment of insulin-independent revertant cell sublines, none of which are tumorigenic.

2. Genotypic analysis revealed 4 revertant cell sublines with retrovirus insertion at different genetic loci. One insertion site is at chromosome 7q11-q21, a band region that does not encode a known gene related to the IGF family.

3. Northern and Western analyses were performed on 23 IGF family-related genes. The results indicated altered expression of IGFIIR and IGFBP3 among 2 revertants studied.

4. Currently, microarray gene expression analysis is being performed on these cell lines in order to identify expression profiles and differentially expressed genes.

REPORTABLE OUTCOMES:

1. AACR 2002 Poster Abstract: Ruttimann, J., Tao, L., Yang, J., Su, Y. Selection and Characterization of Insulin-Independent Cell Sublines from the Chromosome 17-mediated Suppressed Insulin-dependent Breast Cancer Cell Line CAL/17.

CONCLUSIONS:

We have demonstrated the following:

1. Southern analysis of retrovirus sequence-specific DNA and sequence analysis of cloned genomic sequences adjacent to the inserted retrovirus revealed four revertant cell sublines with retrovirus insertion at unique genetic loci. One insertion site is at chromosome 7q11-q21, a band region that does not encode a known gene related to the IGF family.

2. The *in vitro* proliferation rates of the revertant cell sublines fell in between the parental CAL51 and suppressed CAL/17 cell lines in the absence of insulin. All the revertants were anchorage-dependent and non-tumorigenic, similar to the suppressed cell line CAL/17.

3. Northern and Western analyses were performed on 23 IGF family-related genes for these three cell lines. The results indicated altered expression of IGFIIR and IGFBP3 among 2 of the studied revertants.

These results imply that these cell lines provide a useful biological resource to study breast cancer transition from insulin-dependence to insulin-independence. Future directions of this project may include microarray analysis of these cell lines with other breast cancer cell lines that express similar or different characteristics in order to discern genes that may contribute to breast cancer progression from insulin-dependence to insulin-independence.

Selection and Characterization of Insulin-Independent Cell Sublines from the Chromosome17-Mediated Suppressed Insulin-dependent Breast Cancer Cell Line CAL/17

Jacqueline Ruttimann, Lian Tao, Jun Yang, Yan Su

During breast cancer development, tumors progress from an insulin-dependent to an insulin-independent state. To facilitate the identification of genetic loci and genes involved in this development, retroviral insertional mutagenesis was used to induce insulin-independent revertant cell sublines from the insulin-dependent cell line CAL/17. CAL/17 was derived from the parental breast cancer cell line CAL51 by introduction of a *neo*-tagged human chromosome 17. CAL 51 is negative in estrogen receptor expression, carries wild-type p53, and displays the tumorigenic phenotype including rapid population doubling time in the absence of insulin, anchorage-independent growth in soft agar, and rapid tumor formation in athymic nude mice. In contrast, CAL/17 exhibits a requirement for insulin in plastic culture, increased population doubling time, loss of anchorage-independent growth and nontumorigenicity. In this study, retroviral particles were transduced into the suppressed cell line CAL/17 to randomly mutate genomic DNA in the cells. The introduced retrovirus and chromosome 17 were selected by L-histidinol and G418, respectively. After two rounds of selection in the absence of insulin, 48 clones were established as insulin-independent revertant cell sublines. Southern analysis of retrovirus sequence-specific DNA and sequence analysis of cloned genomic sequences adjacent to the inserted retrovirus revealed four revertant cell sublines with retrovirus insertion at unique genetic loci. One insertion site is at chromosome 7q11-q21, a band region that does not encode a known gene related to the IGF family. The *in vitro* proliferation rates of the revertant cell sublines fell in between the parental CAL51 and suppressed CAL/17 cell lines in the absence of insulin. All the revertants were anchorage-dependent and non-tumorigenic, similar to the suppressed cell line CAL/17. Northern and Western analyses were performed on 23 IGF family-related genes for these three cell lines. The results indicated altered expression of IGFIIR and IGFBP3 among 2 of the studied revertants. Currently, microarray gene expression analysis is being performed on these cell lines in order to identify expression profiles and differentially expressed genes. These cell lines provide a useful biological resource to study breast cancer transition from insulin-dependence to insulin-independence.